American Journal of Hematology 53:22–29 (1996)

Mechanism of Intravascular Hemolysis in Paroxysmal Nocturnal Hemoglobinuria (PNH)

Hideki Nakakuma

The Second Department of Internal Medicine, Kumamoto University School of Medicine, Kumamoto, Japan

Paroxysmal nocturnal hemoglobinuria (PNH) hemolysis requires both intravascular complement activation and affected erythrocytes susceptible to complement. This susceptibility is explained by a deficiency in complement regulatory membrane proteins that are attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor. Affected cells lack a series of GPI-anchored membrane proteins with various functions. The lack is caused by a synthetic defect of the anchor due to an impaired transfer of *N*-acetylglucosamine to phosphatidylinositol which is an early metabolic precursor in the anchor synthesis. Moreover, *PIG-A* gene responsible for the membrane defect was recently cloned. Further, a possible mechanism of complement activation has been proposed, especially for an infection-induced hemolytic precipitation which is clinically crucial. Thus, the molecular events, leading to intravascular hemolysis characteristic of PNH, has been virtually clarified. Next major concern is the nature of *PIG-A:* How does *PIG-A* explain the complex pathophysiology of PNH which exhibits various clinical manifestations?

Key words: paroxysmal nocturnal hemoglobinuria, hemolysis, complement regulatory proteins, glycosylphosphatidylinositol anchor, *PIG-A*

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) was first described clinically by Gull in 1866 and was named by Enneking in 1928 [1]. PNH is now regarded as an acquired stem cell disorder of a clonal nature that manifests such characteristic clinical features as intravascular hemolysis, venous thrombosis, bone marrow hypoplasia, and frequent episodes of infection [1,2]. Leukemic development is rarely observed [2]. Hemolysis is a major clinical feature, and is used for the diagnosis of PNH. Association with both hemoglobinuria and hemosiderinuria indicates that PNH hemolysis is intravascular. The hemolysis is clinically characterized as chronic mild hemolysis, nocturnal exacerbations, and hemolytic precipitation induced by infection, transfusion, and operation [1,2]. Hemolysis often causes anemia, and hemolytic exacerbation infrequently leads to the damage of such organs as the kidney. Much attention has therefore been paid to hemolysis. The hemolytic anemia is often treated with steroids and by transfusion with washed normal erythrocytes [2]. Only bone marrow transplantation, which is recommended for PNH patients with severe hypoplasia of bone marrow, may replace the affected clone with a normal clone and consequently abolish the manifestations [2,3]. Recent advances have clarified the entire molecular mechanism of the hemolysis. This review is thus limited to the pathogenesis of PNH hemolysis, although there are other important manifestations such as thrombosis and bone marrow hypoplasia that are major causes of death.

MEMBRANE DEFECTS OF PNH CELLS

Susceptibility to Autologous Complement

When complement is activated by antibody or exposure to low pH (Ham's acidified serum test) or low ionic strength (sugar-water test), PNH erythrocytes undergo hemolysis. Inactivation of complement abolishes the hemolysis. The hemolysis of erythrocytes from PNH patients requires far less complement than that required for the hemolysis of normal erythrocytes, on in vitro treat-

Received for publication January 18, 1996; accepted March 6, 1996.

Address reprint requests to Hideki Nakakuma, The Second Department of Internal Medicine, Kumamoto University School of Medicine, Honjo 1-1-1, Kumamoto 860, Japan.

ment with both anti-erythrocyte antibody and complement [1]. Thus, the critical role played by activated complement and the increased sensitivity of affected erythrocytes to complement are clear in the hemolysis. According to the in vitro sensitivity to antibody-initiated complement-mediated hemolysis, erythrocytes in patients with PNH were classified into three groups: I, normal or almost normal sensitivity; II, moderately sensitive cells; III, markedly sensitive cells [4]. PNH III erythrocytes required as little as 1/25 the concentration of human serum for lysis compared to normal erythrocytes; group II cells required from 1/10 to 1/15 the concentration. Classification of the affected cells has since helped to define the characteristics of PNH cells. The proportion of red cells that are sensitive to complement varies among patients with PNH. The heterogeneous population of differentially sensitive cells and the degree of complement activation in vivo was presumed to explain the various grades of clinical hemolysis.

Disappearance of Complement Regulatory Membrane Proteins

In general, normal tissues are resistant to autologous and homologous complement. However, a greater amount of C3 is fixed to the PNH erythrocyte membrane than to the normal erythrocyte membrane [5]. The C3 fixation and complement susceptibility of PNH erythrocytes indicate the presence of a complement regulatory system in the normal erythrocyte membrane and its absence in PNH cells. Indeed, a 70-kDa membrane protein with complement regulatory activity has been purified from normal erythrocytes [6]. The protein was named decay-accelerating factor (DAF), since it regulates complement activation by inhibiting the formation of or by accelerating the decay of complement complexes with C3/C5 convertase activity in both the classical and the alternative pathways. It has been subsequently shown that DAF is absent in the membranes of affected erythrocytes, leukocytes, and platelets [7]. Moreover, DAF deficiency in PNH is restricted to the blood cells alone. This finding is consistent with the nature of PNH as a stem cell disorder. Similarly to PNH erythrocytes, normal erythrocytes treated with anti-DAF antibody show increased formation of C3 convertase on the membrane, indicating that DAF is involved in the complement regulatory membrane activity [8]. Purified DAF is easily incorporated, restoring complement regulatory activity to a considerable extent in the membranes of affected erythrocytes [8]. Accordingly, it was realized that DAF deficiency causes the loss of complement regulatory activity.

The incorporation of DAF, however, did not normalize the abnormal sensitivity of affected erythrocytes to complement. The most sensitive PNH erythrocytes (PNH III cells) were found to be susceptible to reactive lysis induced by cobra venom factor [9]. Cobra venom factor is a potent activator of the alternative complement pathway, i.e., the factor forms a stable C5 convertase in the alternative pathway and promotes the formation of membrane attack complex (MAC, $C5b\text{-}8(C9)_{1\text{-}18}$). The MAC formation is not regulated by DAF. Incubation in whole serum with cobra venom factor induced striking hemolysis of PNH III cells, but no hemolysis in less sensitive erythrocytes (PNH II cells) or normal erythrocytes. Moreover, C9 binding and poly C9 formation were reported to be abnormally high on PNH cell membranes [9]. These findings suggested that PNH III cells are deficient in inhibitory activity in regard to MAC formation. Membrane proteins with a distinct molecular weight of 20-kDa and potent complement regulatory activity were then found in human erythrocytes [10–12]. This protein blocked C9 polymerization by inhibiting the binding of C9 to C5b-8. Together with other Ly-6-like membrane proteins, i.e., MEM-43 antigen, YTH53.1 leukocyte antigen, and H19, a 19-kDa human erythrocyte antigen, these proteins were shown to be identical, and were designated CD59. CD59, as well as DAF, were also shown to be deficient in the membranes of affected blood cells [11–14]. The incorporation of purified CD59 into the affected erythrocyte membrane restored the resistance to complement to almost the control level [12]. A congenital deficiency of CD59 induced a hemolytic syndrome similar to PNH [15], whereas patients with a congenital deficiency of DAF did not show obvious hemolysis [16]. In PNH hemolysis, the lack of CD59 appears more critical pathophysiologically than the lack of DAF.

Prior to the identification of CD59, but after the identification of DAF, two erythrocyte membrane proteins with complement regulatory activity were identified: a 65-kDa protein that could bind human C8 (C8bp) and a 38-kDa protein (HRF). In contrast to the action of DAF, C8bp did not accelerate the decay of C3 convertase. HRF inhibited the expression of homologous complement transmembrane channels. Treatment with antibody to HRF enhanced the hemolysis of homologous erythrocytes with C5b-9. The antibody did not affect C5b-7 uptake, but affected C9 binding to the target cell membrane. The detection of a 65-kDa protein with anti-HRF antibody suggested a close correlation of HRF with C8bp. PNH cells were deficient in C8bp and HRF [17,18]; however, the identity of these two proteins awaits further molecular characterization.

Lack of Glycosylphosphatidylinositol (GPI)-Anchored Proteins

The defect in multiple proteins of the stromal fabric has been recognized as a pathognomonic feature of PNH cells (Table I). Regarding the molecular nature of the missing proteins, DAF [8] and alkaline phosphatase [19] were readily incorporated into the erythrocyte membrane or into lipid vesicles, indicating their amphipathic proper-

ties. Treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) abolished the incorporation [19]. Later, acetylcholinesterase was shown to be bound by its carboxyl terminus to the carbohydrate chain that was covalently linked to phosphatidylinositol in the outer leaflet of the biomembrane (Fig. 1A) [20]. This glycolipid structure was designated a GPI anchor and initially identified in studies of the rodent membrane glycoprotein Thy-1 and the variant surface glycoprotein (VSG) on the membrane of the parasitic protozoan *Trypanosoma brucei* [21,22]. Similarly to acetylcholinesterase, DAF was soon shown to be attached to the membrane via the anchor [23]. Subsequently, the membrane proteins missing in PNH cells were virtually all proven to be linked with the anchor. The attachment of membrane proteins to the GPI anchor is defined by a signal sequence of about 15–20 hydrophobic amino acid residues at the extreme C-terminus of primary translation products [22]. The number of GPI-anchored membrane proteins thus far identified has increased to over 70. Approximately 30 GPI-anchored membrane proteins have been detected in human cells, and 15 of them are missing from PNH cells (Table I). As to the functional aspects, the defective molecules include enzymes, surface antigens, complement regulatory proteins, and receptors. The membrane defect is detectable by flow cytometry with monoclonal antibodies against GPI-anchored proteins [24]. Flow cytometry can directly identify the missing proteins, the cell lineage, and the population of affected cells, whereas Ham's test indirectly detects affected erythrocytes by complement-mediated hemolysis. These advantages have hastened the application of flow cytometry for the diagnosis of PNH. Flow cytometry has now been used to evaluate hematopoiesis by PNH stem cell [25], has disclosed the frequent occurrence of the PNH clone in patients with aplastic anemia [26], and has contributed to a classification of the clinical stages of PNH [27]. For the identification of PNH cells, two-color flow cytometry with antibodies to DAF and CD59 is now considered to be most reliable, partly because there are reports of deficiencies in a single GPI-anchored protein [15,16].

GPI-Anchor Defect

Contrary to the deficiency in membrane DAF and neutrophil alkaline phosphatase, the expression of their genes was shown to be intact [28,29]. In fact, DAF protein was shown to be produced by PNH cells [30]. The structural determination of GPI-anchors of such membrane proteins as Thy-1 and VSG has revealed that the proteins share a common core structure in the glycolipid anchor: ethanolamine phosphate-mannose(α1-2)mannose(α1-6)mannose(α1-4)glucosamine(α1-6)phosphatidylinositol (PI) (Fig. 1A) [21,22]. The lack of multiple membrane proteins thus appeared to be attributable to a GPI-anchoring defect. Indeed, a synthetic defect of the anchor was demonstrated in PNH granulocytes in 1992 [31]. This finding accelerated the characterization of the interruption site in the anchor synthesis.

Interruption Site in the Synthesis of the GPI-Anchor

Six recessive mutant cell lines which synthesize Thy-1 but lack its GPI-anchoring were discriminated according to their interruption sites in the synthesis of GPI anchor [32]. Structural analysis of the anchor precursors in the mutants has contributed to the definition of the synthetic pathway of GPI anchor in mammalian cells [33]. Eventually, the anchor precursor assembly in murine cells was shown to be basically the same as that in Trypanosoma (Fig. 1B) [22]. First, *N*-acetylglucosaminyl PI (GlcNAc-PI) is synthesized by the incorporation of *N*-acetylglucosamine (GlcNAc) to PI from a sugar nucleotide, uridine diphosphate (UDP)-GlcNAc. This reaction is followed by the rapid deacetylation of the GlcNAc residue and the subsequent acylation of the inositol residue, to produce glucosaminyl (GlcN)-PI with acylated inositol [22,34]. To the product, three mannoses are sequentially transferred from 3 dolichol phosphate-mannoses. Finally, ethanolamine phosphate, supplied from phosphatidylethanolamine (PE), is affixed to the non-reducing carbohydrate end of the mannosylated GlcN-PI. Soon, the anchor is linked to the carboxyl terminus of membrane proteins in the endoplasmic reticulum, possibly by a transamidase. Afterwards, the nascent GPI-anchored proteins are transferred to the Golgi apparatus to further undergo glycosylation of the protein moiety and modification of the anchor moiety. The regulation and physiological significance of the anchor modification remain to be clarified. Next, to identify early biochemical intermediates in the synthesis of the mammalian GPI anchor, an in vitro assay system that had been developed to study the assembly of the VSG GPI-anchor [35] was employed. Comparative assays of reactions in PNH and normal cells showed that mannosylated GlcN-PI was not present in PNH cells [36]. It was also found that PI was produced normally by PNH granulocytes and PNH-T cells, whereas neither GlcNAc-PI, GlcN-PI, mannosylated GlcN-PI, nor GPI anchor were normally produced by PNH cells [34]. In contrast, all these glycolipids were produced in normal control cells. Thus, the interruption site was shown biochemically to inhibit the incorporation of GlcNAc from UDP-GlcNAc to PI, an early step of the anchor biosynthesis (Fig. 1B). Regarding the interruption site, consistent results were obtained independently and almost simultaneously by biochemical analysis [37] and by cell-fusion analysis [38,39]. Of note, the inhibition was not always complete [34,40]. The primary lesion was further confirmed by cloning of the responsible gene, *PIG-A*.

TABLE I. Proteins Missing From PNH Blood Cells

GPI-anchored proteins	References
Complement regulatory proteins	
CD55 (DAF)	[7]
CD59 (MACIF, MIRL, HRF20)	[11,12]
Receptors	
CD14 (Endotoxin-binding protein)	[61]
CD16 (Type III low-affinity Fc receptor for IgG, FcγR III)[a]	[62]
CD87 (Urokinase-type plasminogen activator receptor, UPAR)	[60]
Enzymes	
Erythrocyte acetylcholinesterase	[63]
Leukocyte alkaline phosphatase	[29,64]
Others	
CD24	[24]
CD48 (Blast-1 antigen)	[65]
CD52 (Campath-1 antigen)	[66]
CD58 (Leukocyte function antigen-3, LFA-3)[a]	[67]
CD66	[68]
CD67	[24]
CLBgran/5 antigen (A 50–80 kDa granulocyte antigen)	[24]
JMH antigen	[69]
Proteins not identified as GPI-anchored	
C8 binding protein (C8bp)/Homologous restriction factor (HRF)	[17,18]
Possibly deficient GPI-anchored proteins	
Folate receptor	[70]
CDw109 (A 175 kDa platelet glycoprotein)	[71]

[a]Proteins that use both GPI and peptide anchors to localize in the membrane.

PIG-A Gene Responsible for the GPI-Anchor Defect

In parallel with the biochemical disclosure of the interruption site in the synthesis of the GPI anchor in PNH cells, a complementary DNA (*PIG-A*) was cloned by expression cloning with class A mutant cells (JY5, a human B-lymphoblastic cell line which is unable to synthesize GlcNAc-PI) [33] as a recipient of cDNA from a HeLa cell library with an Epstein-Barr virus vector [41]. The cDNA contains an open reading frame of 1,452 bp, coding a protein of 484 amino acid residues. Among the three mutants (classes A, C, and H) that are unable to produce GlcNAc-PI [33], there was preliminary evidence that the B cell lines established from Japanese patients with PNH belonged to class A, according to complementation analysis by somatic cell hybridization with GPI-deficient murine mutant cell lines [39]. As expected, the transfection of *PIG-A* cDNA into the affected PNH cell lines restored the expression of the missing GPI-anchored membrane proteins on the affected cells [42]. *PIG-A* was then named after the correction of phosphatidylinositol glycan (PIG) synthesis in the class A mutant cells. A missing transcript, a low level transcript, and an abnormally spliced transcript of *PIG-A* were subsequently demonstrated in each of the PNH B-cell lines [42] and in affected granulocytes as well [43]. *PIG-A* was expressed normally in intact blood cells (GPI-anchor-positive lymphocytes and granulocytes) obtained from PNH patients. It was thus demonstrated that a somatic mutation of *PIG-A* occurred in the PNH clone. By fluorescence in situ hybridization with biotinylated genomic *PIG-A* DNA probes, *PIG-A* was mapped on the X chromosome (Xp22.1) [42]. In the PNH cell lines, a normal sized *PIG-A* transcript was not detected, whereas a normal *PIG-A* allele was detected, indicating the dominant expression of abnormal *PIG-A* in PNH cells. The localization of *PIG-A* on the X chromosome may explain this dominant expression of the mutated gene in PNH. Namely, pathologic mutations of *PIG-A* occurred in the allele in the active X chromosome. Although the PNH phenotype has been shown to be recessive [44], the mutation of *PIG-A* in a single allele is sufficient to induce a class A defect (PNH phenotype), when the mutated allele is on the active X chromosome. In support of this idea, during the establishment of the Thy-1-negative mutant cell lines, class A mutants were most frequently obtained. Altered expression of *PIG-A* was also detected in the affected blood cells of all of 15 patients with PNH examined [43]. These findings strongly suggest that a mutation in *PIG-A* impairs the expression of the GPI anchor in PNH. The *PIG-A* mutation, and, especially, different mutations in *PIG-A*, were further demonstrated in patients with PNH in Europe [45], North America [46], and Thailand [47]. Interestingly, multiple mutations that spread throughout the entire *PIG-A* coding regions have been found [45,46,48,49], and multiple clones with different mutations were also detected in a patient with PNH [49]. Among multiple clones with *PIG-A* mutations, a single clone which pre-

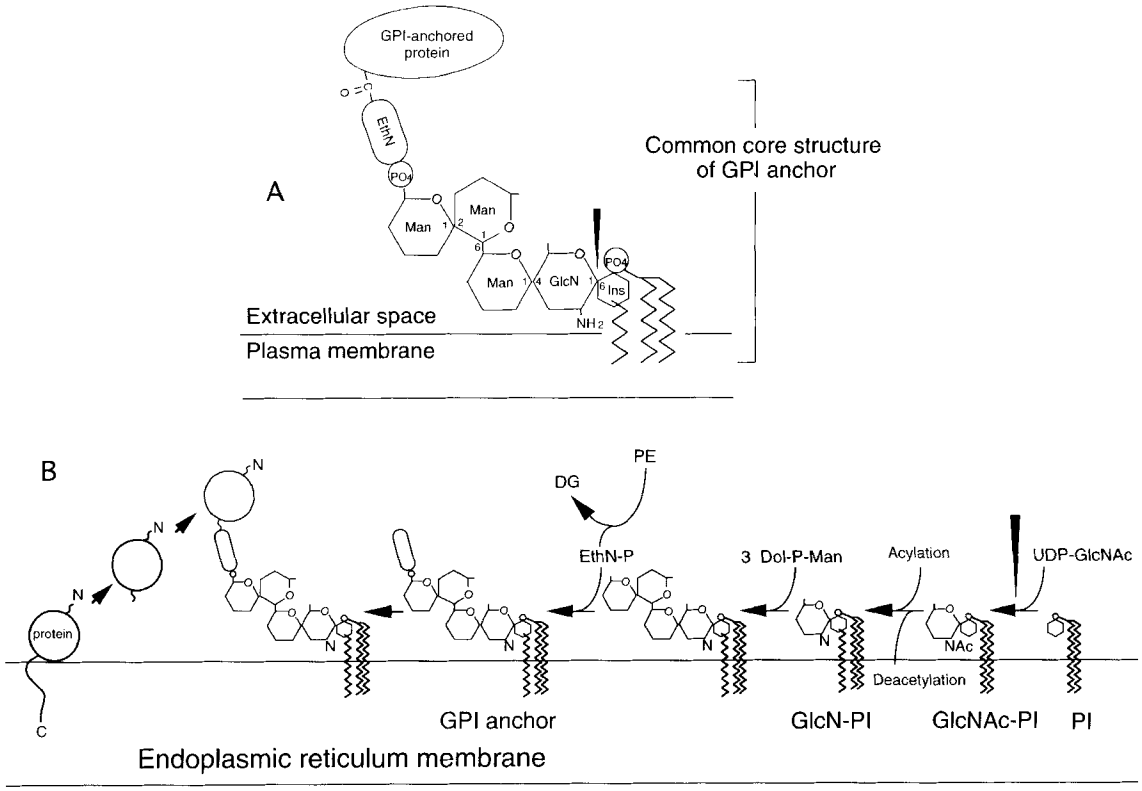

Fig. 1. Structure and biosynthesis of a GPI-anchored membrane protein. A: The core structure of the anchor. The precise site of acylation of the inositol residue of the anchor has not yet been identified. EthN, ethanolamine; PO4, phosphate; Man, mannose; GlcN, glucosamine; Ins, inositol. B: Synthetic pathway of the anchor and the transfer of protein to the anchor. PI, phosphatidylinositol; GlcNAc-PI, *N*-acetylglucosaminyl phosphatidylinositol; GlcN-PI, glucosaminyl phosphatidylinositol; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine; Dol-P-Man, dolichol phosphate-mannose; PE, phosphatidylethanolamine; DG, diacylglycerol; EthN-P, ethanolamine phosphate; C, carboxyl terminal hydrophobic signal peptide of primary translation product. Vertical solid arrows (▼) indicate the interruption site in the synthesis of GPI-anchor in PNH cells.

dominantly proliferates might be responsible for the clinical manifestations.

Other Membrane Defects

Abnormal expression was further noted in gangliosides and glycophorin A of the membrane of PNH erythrocytes [34,50,51]. Change of membrane glycoconjugates was also found in other rare hematological diseases, such as Tn syndrome and hereditary erythroblastic multinuclearity with positive acidified serum test (HEMPAS) [51,52]. PNH and Tn syndrome share a somatic mutation of hematopoietic stem cells and the infrequent development of leukemia. PNH and HEMPAS erythrocytes are markedly susceptible to lysis by antibody plus complement. Based on these similarities, a greater attempt is currently being made to relate the membrane alteration to the various clinical manifestations of PNH. Indeed, it is suggested that the altered expression of membrane glycoconjugates is, in part, involved in the increased hemolysis of PNH erythrocytes by a mechanism independent of the lack of complement regulatory proteins [51,53]. The pathophysiologic significance of the membrane alteration is still an area for further investigation.

INTRAVASCULAR ACTIVATION OF COMPLEMENT

For PNH hemolysis, intravascular complement activation is indispensable, besides the membrane defects of affected erythrocytes. Persistent mild PNH hemolysis may be explained by a low level of nonspecific spontaneous activation of the alternative complement pathway. Of the types of PNH hemolysis, episodic hemolysis, which is often precipitated by infections, is distinct because of the rapid induction of serious anemia and the damage to critical organs. A potential trigger of the complement activation leading to the infection-associated precipitation of hemolysis was recently proposed [54]. In short, the expression of cryptantigens, which are exposed by the infection-associated release of non-reducing terminal sugars from membrane glycoconjugates [52], induced the immune reaction with natural antibodies [52,55], leading to complement activation on the erythrocyte membranes,

and resulting in the selective and potent intravascular hemolysis of cryptantigen-positive PNH erythrocytes. To establish the clinical relevance of cryptantigen-mediated hemolytic precipitation, however, a large scale investigation of PNH patients with hemolytic attack is needed. Ascorbic acid (vitamin C) is an essential vitamin that is generally accepted as harmless. However, exacerbation of complement-mediated intravascular hemolysis was reported in patients with PNH after taking drinks enriched with ascorbic acid [56]. After discontinuing the use of the drinks, the hemolysis ceased. Attention should therefore be paid to these untoward effects, although the precise mechanism is unknown.

FUTURE DIRECTIONS

Characterization of the structural and functional properties of the coding protein of the *PIG-A* gene is required to explain the precise mechanism by which the protein restores the interruption of GPI anchor synthesis (impaired transfer of GlcNAc to PI) in affected cells. Urgent problems to be addressed are: whether the *PIG-A* gene encodes GlcNAc transferases; what determines the difference between the complete and incomplete synthesis of the anchor in *PIG-A* mutated cells [34,40]; and whether *PIG-A* repairs the altered expression of the carbohydrate moiety of membrane glycoconjugates other than the anchor in PNH cells [50,51]. The mechanism whereby the *PIG-A* mutation is induced is another important area for further investigation, since multiple mutations have been noted [45–49].

The next major concern is the pathogenesis of other clinical manifestations, such as bone marrow hypoplasia and thrombosis, which are major causes of death in PNH. Above all, the relation of the clinical manifestations with the *PIG-A* mutation is of interest. Regarding the hypoplasia, the frequent detection of affected cells with PNH phenotype in patients with aplastic anemia has affirmed the close association between PNH and this disease [24,26,27,57]. The spectrum of the *PIG-A* mutations in affected cells was not obviously different in patients with primary PNH compared to the spectrum in those with previous AA [48]. To elucidate the complex pathophysiology of the hypoplasia, it is necessary to further clarify the nature of the PNH clone. Interestingly, the population of affected cells is very low when the hematopoietic capability of PNH bone marrow is normal. In contrast, PNH cells become dominant in the hypoplastic marrow and PNH becomes manifest. The defect in PNH clone may render affected cells insensitive to the suppressive process of hematopoiesis in bone marrow characteristic of aplastic anemia [58]. Besides this survival advantage, PNH clone has been speculated to exhibit a growth advantage over the normal clone in vivo [49]. Whether the PNH clone exhibits any neoplastic features and whether the *PIG-A* mutation is responsible for the growth or survival properties are thus important areas. A recent study has shown that the mutated *PIG-A* genome exhibited no additional gross genetic changes in the leukemic clone that evolved in a patient with PNH [59].

There are some important attempts to attribute the thrombotic tendency in PNH to the *PIG-A* mutation [14,60]; i.e., the deficiency of CD59 which accounts for the hemolysis may also be responsible for the thrombotic tendency in PNH. In brief, intravascular hemolysis may initiate or promote the coagulation process. It is suggested that the deficiency of CD59 on platelets leads to the generation of thrombin [14]. This generation may partly explain the high incidence of venous thrombosis. Thrombosis may be further enhanced by the absence of the urokinase-type plasminogen activator receptor (UPAR) which is attached by GPI-anchor to the leukocyte membrane [60]. Plasminogen activator binds the UPAR and converts plasminogen to plasmin. Plasmin then cleaves fibrin to dissolve the clot. It is thus possible that absence of the UPAR keeps the clot stable and consequently promotes the formation of thrombi in patients with PNH.

Subsequent to the recent clarification of the molecular mechanism which causes the intravascular hemolysis characteristic of PNH, it can also be expected that the mystery of the molecular events leading to dysplastic hematopoiesis and venous thrombosis will soon be unraveled.

ACKNOWLEDGMENTS

The author thanks Drs. T. Kawaguchi, K. Horikawa, M. Hidaka, S. Nagakura, N. Iwamoto, T. Kagimoto, and K. Takatsuki for their advice and help, and thanks F. Inukai for her secretarial assistance.

REFERENCES

1. Rosse WF: Evolution of clinical understanding: Paroxysmal nocturnal hemoglobinuria as a paradigm. Am J Hematol 42:122, 1993.
2. Lee GR: Paroxysmal nocturnal hemoglobinuria. In Lee GR, Bithell TC, Foerster J, Athens JW, Lukens JN (eds): "Wintrobe's Clinical Hematology." Ed 9. Vol 1. Philadelphia: Lea & Febiger, 1993, p 1232.
3. Kawahara K, Witherspoon RP, Storb R: Marrow transplantation for paroxysmal nocturnal hemoglobinuria. Am J Hematol 39:283, 1992.
4. Rosse WF, Dacie JV: Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. I. The sensitivity of PNH red cells to lysis by complement and specific antibody. J Clin Invest 45:736, 1966.
5. Parker CJ, Baker PJ, Rosse WF: Increased enzymatic activity of the alternative pathway convertase when bound to the erythrocytes of paroxysmal nocturnal hemoglobinuria. J Clin Invest 69:337, 1982.
6. Nicholson-Weller A, Burge J, Fearon DT, Weller PF, Austen KF: Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. J Immunol 129:184, 1982.
7. Nicholson-Weller A, Spicer DB, Austen KF: Deficiency of the complement regulatory protein, "decay-accelerating factor," on membranes

of granulocytes, monocytes, and platelets in paroxysmal nocturnal hemoglobinuria. N Engl J Med 312:1091, 1985.
8. Medof ME, Kinoshita T, Silber R, Nussenzweig V: Amelioration of lytic abnormalities of paroxysmal nocturnal hemoglobinuria with decay-accelerating factor. Proc Natl Acad Sci USA 82:2980, 1985.
9. Hu VW, Nicholson-Weller A: Enhanced complement-mediated lysis of type III paroxysmal nocturnal hemoglobinuria erythrocytes involves increased C9 binding and polymerization. Proc Natl Acad Sci USA 82:5520, 1985.
10. Sugita Y, Nakano Y, Tomita M: Isolation from human erythrocytes of a new membrane protein which inhibits the formation of complement transmembrane channels. J Biochem 104:633, 1988.
11. Okada N, Harada R, Fujita T, Okada H: A novel membrane glycoprotein capable of inhibiting membrane attack by homologous complement. Int Immunol 1:205, 1989.
12. Holguin MH, Fredrick LR, Bernshaw NJ, Wilcox LA, Parker CJ: Isolation and characterization of a membrane protein from normal human erythrocytes that inhibits reactive lysis of the erythrocytes of paroxysmal nocturnal hemoglobinuria. J Clin Invest 84:7, 1989.
13. Nagakura S, Nakakuma H, Horikawa K, et al.: Expression of decay-accelerating factor and CD59 in lymphocyte subsets of healthy individuals and paroxysmal nocturnal hemoglobinuria patients. Am J Hematol 43:14, 1993.
14. Wiedmer T, Hall SE, Ortel TL, Kane WH, Rosse WF, Sims PJ: Complement-induced vesiculation and exposure of membrane prothrombinase sites in platelets of paroxysmal nocturnal hemoglobinuria. Blood 82:1192, 1993.
15. Yamashina M, Ueda E, Kinoshita T, et al.: Inherited complete deficiency of 20-kilodalton homologous restriction factor (CD59) as a cause of paroxysmal nocturnal hemoglobinuria. N Engl J Med 323:1184, 1990.
16. Telen MJ, Hall SE, Green AM, Moulds JJ, Rosse WF: Identification of human erythrocyte blood group antigens on decay accelerating factor (DAF) and an erythrocyte phenotype negative for DAF. J Exp Med 167:1993, 1988.
17. Hänsch GM, Schönermark S, Roelcke D: Paroxysmal nocturnal hemoglobinuria type III. Lack of an erythrocyte membrane protein restricting the lysis of C5b-9. J Clin Invest 80:7, 1987.
18. Zalman LS, Wood LM, Frank MM, Müller-Eberhard HJ: Deficiency of the homologous restriction factor in paroxysmal nocturnal hemoglobinuria. J Exp Med 165:572, 1987.
19. Low MG, Zilversmit DB: Role of phosphatidylinositol in attachment of alkaline phosphatase to membranes. Biochemistry 19:3913, 1980.
20. Roberts WL, Santikarn S, Reinhold VN, Rosenberry TL: Structural characterization of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase by fast atom bombardment mass spectrometry. J Biol Chem 263:18776, 1988.
21. Ferguson MAJ, Williams AF: Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. Annu Rev Biochem 57:285, 1988.
22. Englund PT: The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. Annu Rev Biochem 62:121, 1993.
23. Medof ME, Walter EI, Roberts WL, Haas R, Rosenberry TL: Decay-accelerating factor of complement is anchored to cells by a C-terminal glycolipid. Biochemistry 25:6740, 1986.
24. van der Schoot CE, Huizinga TWJ, van't Veer-Korthof ET, Wijmans R, Pinkster J, von dem Borne AEGKr: Deficiency of glycosyl-phosphatidylinositol-linked membrane glycoproteins of leukocytes in paroxysmal nocturnal hemoglobinuria, description of a new diagnostic cytofluorometric assay. Blood 76:1853, 1990.
25. Iwamoto N, Kawaguchi T, Nagakura S, et al.: Markedly high population of affected reticulocytes negative for decay-accelerating factor and CD59 in paroxysmal nocturnal hemoglobinuria. Blood 85:2228, 1995.
26. Nakakuma H, Nagakura S, Iwamoto N, et al.: Paroxysmal nocturnal hemoglobinuria clone in bone marrow of patients with pancytopenia. Blood 85:1371, 1995.
27. Nakakuma H, Kawaguchi T, Horikawa K, et al.: Proposal for a classification of the clinical stages of paroxysmal nocturnal hemoglobinuria. Blood 86:2051, 1995.
28. Stafford HA, Tykocinski ML, Lublin DM, et al.: Normal polymorphic variations and transcription of the decay accelerating factor gene in paroxysmal nocturnal hemoglobinuria cells. Proc Natl Acad Sci USA 85:880, 1988.
29. Rambaldi A, Terao M, Bettoni S, et al.: Differences in the expression of alkaline phosphatase mRNA in chronic myelogenous leukemia and paroxysmal nocturnal hemoglobinuria polymorphonuclear leukocytes. Blood 73:1113, 1989.
30. Carothers DJ, Hazra SV, Andreson SW, Medof ME: Synthesis of aberrant decay-accelerating factor proteins by affected paroxysmal nocturnal hemoglobinuria leukocytes. J Clin Invest 85:47, 1990.
31. Mahoney JF, Urakaze M, Hall S, et al.: Defective glycosylphosphatidylinositol anchor synthesis in paroxysmal nocturnal hemoglobinuria granulocytes. Blood 79:1400, 1992.
32. Hyman R: Cell-surface-antigen mutants of haematopoietic cells. Tools to study differentiation, biosynthesis and function. Biochem J 225: 27, 1985.
33. Stevens VL, Raetz CRH: Defective glycosyl phosphatidylinositol biosynthesis in extracts of three Thy-1 negative lymphoma cell mutants. J Biol Chem 266:10039, 1991.
34. Hidaka M, Nagakura S, Horikawa K, et al.: Impaired glycosylation of glycosylphosphatidylinositol-anchor synthesis in paroxysmal nocturnal hemoglobinuria leukocytes. Biochem Biophys Res Commun 191: 571, 1993.
35. Masterson WJ, Doering TL, Hart GW, Englund PT: A novel pathway for glycan assembly: Biosynthesis of the glycosylphosphatidylinositol anchor of the trypanosome variant surface glycoprotein. Cell 56: 793, 1989.
36. Hirose S, Ravi L, Prince GM, et al.: Synthesis of mannosylglucosaminylinositol phospholipids in normal but not paroxysmal nocturnal hemoglobinuria cells. Proc Natl Acad Sci USA 89:6025, 1992.
37. Hillmen P, Bessler M, Mason PJ, Watkins WM, Luzzatto L: Specific defect in *N*-acetylglucosamine incorporation in the biosynthesis of the glycosylphosphatidylinositol anchor in cloned cell lines from patients with paroxysmal nocturnal hemoglobinuria. Proc Natl Acad Sci USA 90:5272, 1993.
38. Armstrong C, Schubert J, Ueda E, et al.: Affected paroxysmal nocturnal hemoglobinuria T lymphocytes harbor a common defect in assembly of *N*-acetyl-D-glucosamine inositol phospholipid corresponding to that in class A Thy-1$^-$ murine lymphoma mutants. J Biol Chem 267: 25347, 1992.
39. Takahashi M, Takeda J, Hirose S, et al.: Deficient biosynthesis of *N*-acetylglucosaminyl-phosphatidylinositol, the first intermediate of glycosyl phosphatidylinositol anchor biosynthesis, in cell lines established from patients with paroxysmal nocturnal hemoglobinuria. J Exp Med 177:517, 1993.
40. Norris J, Sharon H, Ware RE, et al.: Glycosyl-phosphatidylinositol anchor synthesis in paroxysmal nocturnal hemoglobinuria: Partial or complete defect in an early step. Blood 83:816, 1994.
41. Miyata T, Takeda J, Iida Y, et al.: The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. Science 259:1318, 1993.
42. Takeda J, Miyata T, Kawagoe K, et al.: Deficiency of the GPI anchor caused by a somatic mutation of the *PIG-A* gene in paroxysmal nocturnal hemoglobinuria. Cell 73:703, 1993.
43. Miyata T, Yamada N, Iida Y, et al.: Abnormalities of *PIG-A* transcripts in granulocytes from patients with paroxysmal nocturnal hemoglobinuria. N Engl J Med 330:249, 1994.
44. Hillmen P, Bessler M, Bungey J, Luzzatto L: Paroxysmal nocturnal hemoglobinuria: Correction of abnormal phenotype by somatic cell hybridization. Somat Cell Mol Genet 19:123, 1993.
45. Bessler M, Mason PJ, Hillmen P, et al.: Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. EMBO J 13:110, 1994.
46. Ware RE, Rosse WF, Howard TA: Mutations within the *Piga* gene in

patients with paroxysmal nocturnal hemoglobinuria. Blood 83:2418, 1994.
47. Pramoonjago P, Wanachiwanawin W, Chinprasertsak S, Pattanapanayasat K, Takeda J, Kinoshita T: Somatic mutations of *PIG-A* in Thai patients with paroxysmal nocturnal hemoglobinuria. Blood 86:1736, 1995.
48. Nafa K, Mason PJ, Hillmen P, Luzzatto L, Bessler M: Mutations in the PIG-A gene causing paroxysmal nocturnal hemoglobinuria are mainly of the frameshift type. Blood 86:4650, 1995.
49. Bessler M, Mason P, Hillmen P, Luzzatto L: Somatic mutations and cellular selection in paroxysmal nocturnal haemoglobinuria. Lancet 343:951, 1994.
50. Nakakuma H, Kawaguchi T, Horikawa K, et al.: Altered expression of gangliosides in erythrocytes of paroxysmal nocturnal hemoglobinuria. J Clin Invest 85:1456, 1990.
51. Parker CJ, Soldato CM, Rosse WF: Abnormality of glycophorin-α on paroxysmal nocturnal hemoglobinuria erythrocytes. J Clin Invest 73:1130, 1984.
52. Judd WJ, Review: Polyagglutination. Immunohematology 8:58, 1992.
53. Horikawa K, Nakakuma H, Nagakura S, et al.: Hemolysis of human erythrocytes is a new bioactivity of gangliosides. J Exp Med 174: 1385, 1991.
54. Nakakuma H, Hidaka M, Nagakura S, et al.: Expression of cryptantigen Th on paroxysmal nocturnal hemoglobinuria erythrocytes in association with a hemolytic exacerbation. J Clin Invest 96:201, 1995.
55. Dacie J: The auto-immune haemolytic anaemias. In Dacie J (ed): "The Haemolytic Anaemias." Ed 3. Vol 3. Edinburgh: Churchill Livingstone, 1992, pp 1–528.
56. Iwamoto N, Kawaguchi T, Horikawa K, et al.: Haemolysis induced by ascorbic acid in paroxysmal nocturnal hemoglobinuria. Lancet 343:357, 1994.
57. Schubert J, Vogt HG, Zielinska-Skowronek M, et al.: Development of the glycosylphosphatidylinositol-anchoring defect characteristic for paroxysmal nocturnal hemoglobinuria in patients with aplastic anemia. Blood 83:2323, 1994.
58. Young NS: The problem of clonality in aplastic anemia: Dr. Dameshek's riddle, restated. Blood 79:1385, 1992.
59. Stafford HA, Nagarajan S, Weinberg JB, Medof ME: PIG-A, DAF and proto-oncogene expression in paroxysmal nocturnal haemoglobinuria-associated acute myelogeneous leukaemia blasts. Br J Haematol 89: 72, 1995.
60. Ploug M, Plesner T, Rønne E, et al.: The receptor for urokinase-type plasminogen activator is deficient on peripheral blood leukocytes in patients with paroxysmal nocturnal hemoglobinuria. Blood 79:1447, 1992.
61. Haziot A, Chen S, Ferrero E, Low MG, Silber R, Goyert SM: The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. J Immunol 141:547, 1988.
62. Selvaraj P, Rosse WF, Silber R, Springer TA: The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal hemoglobinuria. Nature 333:565, 1988.
63. Auditore JV, Hartmann RC: Paroxysmal nocturnal hemoglobinuria. II. Erythrocyte acetylcholinesterase defect. Am J Med 27:401, 1959.
64. Beck WS, Valentine WN: Biochemical studies on leucocytes. II. Phosphatase activity in chronic lymphatic leucemia, acute leucemia, and miscellaneous hematologic conditions. J Lab Clin Med 38:245, 1951.
65. Schubert J, Alvarado M, Uciechowski P, et al.: Diagnosis of paroxysmal nocturnal hemoglobinuria using immunophenotyping of peripheral blood cells. Br J Haematol 79:487, 1991.
66. Nagakura S, Kawaguchi T, Horikawa K, et al.: A deficiency in CDw52 (Campath-1 antigen) of paroxysmal nocturnal hemoglobinuria lymphocytes. Blood 82:3790, 1993.
67. Selvaraj P, Dustin ML, Silber R, Low MG, Springer TA: Deficiency of lymphocyte function-associated antigen 3 (LFA-3) in paroxysmal nocturnal hemoglobinuria. Functional correlates and evidence for a phosphatidylinositol membrane anchor. J Exp Med 166:1011, 1987.
68. Mayne KM, Pulford K, Jones M, et al.: Antibody By114 is selective for the 90 kD PI-linked component of the CD66 antigen: A new reagent for the study of paroxysmal nocturnal haemoglobinuria. Br J Haematol 83:30, 1993.
69. Bobolis KA, Moulds JJ, Telen MJ: Isolation of the JMH antigen on a novel phosphatidylinositol-linked human membrane protein. Blood 79:1574, 1992.
70. Antony A: The biological chemistry of folate receptors. Blood 79: 2807, 1992.
71. Smith JW, Hayward CPM, Horsewood P, Warkentin TE, Denomme GA, Kelton JG: Characterization and localization of the $Gov^{a/b}$ alloantigens to the glycosylphosphatidylinositol-anchored protein CDw109 on human platelets. Blood 86:2807, 1995.